Carbon Assimilation Pathways during Growth of *Pseudomonas* AM1 on Methylamine and *Pseudomonas* MS on Methylamine and Trimethylsulphonium Salts

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(Received 24 March 1972; revised 2 May 1972)

SUMMARY

A study was made of the incorporation of carbon from [^{14}C]methylamine by cultures of *Pseudomonas* AM1 growing on methylamine. The distribution of radioactivity within the non-volatile constituents of the ethanol-soluble fractions of the bacteria, after incubation for periods of up to 38 s, was analysed by chromatography and autoradiography. Most of the radioactivity fixed at the earliest times of sampling appeared in serine, phosphorylated compounds and malate. These results contrasted with previous studies of the incorporation of labelled growth substrates by cultures of *Pseudomonas* MS growing on methylamine or trimethylsulphonium salts and correlated with the specific activities of hydroxypyruvate reductase, phosphoenolpyruvate carboxylase, *N*-methyl glutamate synthetase and γ -glutamylmethylamide synthetase in bacterial extracts. No hydroxypyruvate reductase was detectable in extracts of *Pseudomonas* MS grown either on methylamine or on trimethylsulphonium nitrate. The assimilation pathway for methylamine in *Pseudomonas* AM1 appears essentially the same as that for methanol and different from that in *Pseudomonas* MS.

INTRODUCTION

The group of bacteria capable of aerobic growth on C_1 compounds includes two species which can grow at the expense of methylamine: *Pseudomonas* AM1 and *Pseudomonas* MS. Growth of *Pseudomonas* AM1 on methanol involves serine as an early product of methanol metabolism (Large, Peel & Quayle, 1961). Growth of *Pseudomonas* AM1 on methylamine has been assumed to involve a similar assimilation pathway to that for methanol because mutants lacking the following enzymes of the serine pathway are unable to grow on either methanol or methylamine: hydroxypyruvate reductase (Heptinstall & Quayle, 1970); serine-glyoxylate aminotransferase (Harder & Quayle, 1971); glycerate kinase (Anthony, Dunstan & Drabble, 1971). By contrast, when *Pseudomonas* MS was grown on methylamine, serine was not found to be an early product of C_1 assimilation; instead, incubation of intact bacteria with radioactive methylamine resulted in the rapid formation of two unusual amino acids: *N*-methylglutamate and γ -glutamylmethylamide (Kung, 1969; Kung & Wagner, 1969). The former compound was first identified as an early product of methylamine incorporation in a related species of *Pseudomonas* by Shaw, Tsai & Stadtman (1966). These investigators also described the enzyme reaction responsible for the synthesis of *N*-methylglutamate. The reaction responsible for the biosynthesis of γ -glutamylmethylamide was described by Kung & Wagner (1969) and both of these enzymes were shown to be

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induced in *Pseudomonas* MS when grown on methylamine (Kung, 1969; Kung & Wagner, 1969).

These results suggest that *Pseudomonas* AMI and *Pseudomonas* MS grow on methylamine by two different assimilatory pathways and call for confirmation by determination of the early products of methylamine assimilation by *Pseudomonas* AMI and comparison of the levels of key enzymes for the incorporation of C_1 compounds in both *Pseudomonas* AMI and *Pseudomonas* MS. This paper records the results of such a study.

METHODS

Growth of the organism

Pseudomonas AMI was grown on methylamine as described by Heptinstall & Quayle (1970). *Pseudomonas* MS was grown as described by Kung & Wagner (1970).

Preparation of tracer solutions

[^{14}C]Methylamine hydrochloride was purchased from the Radiochemical Centre, Amersham, Buckinghamshire. It was purified before use by vacuum distillation of [^{14}C]-methylamine released from the hydrochloride by treatment with sodium hydroxide.

Incorporation of [^{14}C]methylamine by whole cells

Cultures of methylamine-grown bacteria in the exponential growth phase were centrifuged and resuspended at 4 mg dry wt/ml in 9 ml of a solution containing 10 mM-Na/K phosphate buffer (pH 7), 10 mM- NH_4Cl and 10 mM-methylamine hydrochloride. The suspension was placed in a cylindrical glass vessel (10 cm $\times$ 2.5 cm diam.) with a sintered glass bottom and was aerated at 20 °C by blowing air through the sintered glass. After 5 min aeration, 1 ml of a solution of [^{14}C]methylamine hydrochloride (6.25 μ mol containing 200 μ Ci of ^{14}C) was added and samples (approx. 1 ml) were withdrawn at suitable time intervals with a hypodermic syringe and squirted into 4 ml of aqueous 95% (v/v) ethanol at room temperature. A control incubation with 1 ml of a boiled bacterial suspension and a proportional volume of tracer solution was performed and subsequently treated in the same way as the samples obtained from the live incubation.

Preparation of samples for chromatography

The ethanolic samples of killed organisms were centrifuged and the resulting precipitates extracted with 0.2 ml of aqueous 20% (v/v) ethanol. The combined supernatants were evaporated to dryness at 40 °C under reduced pressure and taken up in 0.2 ml of water. Each sample was transferred to the top of a column (2 cm $\times$ 0.5 cm diam.) of Amberlite CG 120 (100–200 mesh: H^+ form) and eluted with 2 ml of 2 M- NH_4OH to displace cationic species of less basicity than $CH_3NH_3^+$ (Shaw *et al.* 1966). The eluates were again evaporated to dryness at 40 °C under vacuum and taken up in 0.2 ml of aqueous 20% (v/v) ethanol.

Chromatography and radioautography

The methods of chromatography, autoradiography and identification of labelled compounds were as described by Large *et al.* (1961), unless specified otherwise.

Succinic, fumaric, citric and isocitric acids were identified by two-dimensional cellulose thin-layer co-chromatography in the system described by Myers & Huang (1969). Citric and isocitric acids were also identified by co-electrophoresis on Whatman 3 MM paper for 45 min at a potential gradient of 40 V/cm in a buffer system consisting of 0.5 M-acetic acid adjusted to pH 4 with pyridine (Gross, 1959). Aspartic acid, glutamic acid, serine and

glycine were separated and identified by electrophoresis under similar conditions as described above except that the following two buffer systems were used: formic acid–water (1:37.5, v/v; pH 2); pyridine–acetic acid–water (200:8:2792, by vol.; pH 6.4).

Measurement of radioactivity

The radioactivity on paper chromatograms was measured with a mica end-window G.M. tube (Large *et al.* 1961), appropriate corrections being made for background counts.

Preparation of bacterial extracts

For the measurement of hydroxypyruvate reductase and phosphoenolpyruvate carboxylase 1 vol. of frozen bacteria was suspended in 3 vol. of 0.05 M-K phosphate buffer, pH 7.0, and treated in an MSE ultrasonic disintegrator (MSE model 60W) for 2 min at 0 °C (power output 60W at 25 kHz). For the measurement of γ -glutamylmethylamide synthetase and *N*-methylglutamate synthetase one volume of frozen bacteria was suspended in three volumes of 0.01 M-K phosphate buffer, pH 7.0, containing: methylamine, 1 mM; Na glutamate, 1 mM; MnCl₂, 1 mM; dithiothreitol, 1 mM; and Na₃ ATP, 1 mM. The presence of these compounds in the buffer during preparation of the extracts stabilizes the activity of γ -glutamylmethylamide synthetase. This suspension was passed twice through a French pressure cell. Unbroken organisms and debris were removed by centrifugation at 20000 g for 30 min.

Enzyme assays

Hydroxypyruvate reductase (EC. 1.1.1.29) was assayed aerobically at pH 4.5 by the method of Large & Quayle (1963). Phosphoenolpyruvate carboxylase (EC. 4.1.1.31) was assayed by the method of Large, Peel & Quayle (1962), which measures the phosphoenolpyruvate-dependent fixation of [¹⁴C]bicarbonate, except that the pH of the assay mixture was 8.6 instead of 7.5. *N*-Methylglutamate synthetase was assayed by the method of Shaw & Stadtman (1970). γ -Glutamylmethylamide synthetase was assayed by a modification of the method of Kung & Wagner (1969). The reaction mixture contained Na glutamate, 10 μ mol; MnCl₂, 5 μ mol; Na₃ATP, 5 μ mol; dithiothreitol, 10 μ mol; [¹⁴C]methylamine, 5 μ mol (0.05 μ Ci/ μ mol); K phosphate buffer, pH 7.0, 100 μ mol; and extract in a total volume of 1.0 ml. The reaction mixture was incubated for 1 h at 37 °C and then 0.1 ml of 30% trichloroacetic acid was added to stop the reaction. The mixture was centrifuged and 0.4 ml of the supernatant solution was applied to a small column (5 $\times$ 25 mm) containing Dowex 50 (H⁺) cation exchange resin. The column was washed with 2 ml of water and the γ -glutamylmethylamide synthesized in the reaction was eluted with 2 ml of 2 N-NH₄OH. The entire 2 ml of the NH₄OH eluate was added to 10 ml of Bray's solution (Bray, 1960) and counted in a liquid scintillation counter. Correction for background and non-specific radioactivity from the column was made by carrying a boiled enzyme control through the entire procedure.

The activities of all the enzymes are expressed in milli-units (mu), where 1 mu catalyses the transformation of 1 nmole of substrate in 1 min.

RESULTS

The incorporation of [¹⁴C]methylamine by intact cells of Pseudomonas AM1

When methylamine-grown *Pseudomonas* AM1 was incubated with radioactive methylamine for short periods of time most of the radioactivity was found in serine at the earliest time period (5 s). Table 1 shows that smaller amounts of radioactivity were found in

Table 1. Incorporation of [14 C]methylamine by *Pseudomonas* AM1 at 20 °C

[14 C]Methylamine hydrochloride (6.25 μ mol; 200 μ Ci) was added at zero time to 10 ml of a suspension of bacteria (4 mg dry wt/ml) in 10 mM-Na/K phosphate buffer, pH 7.0, containing 10 mM-NH $_4$ Cl and 10 mM-methylamine hydrochloride. The suspension was gassed with air in a cylindrical vessel (10 cm $\times$ 2.5 cm diam.) with a sintered glass bottom. Samples (approx. 1 ml) were removed at intervals into 4 ml of aqueous 95% (v/v) ethanol and centrifuged; the pellets were extracted with 0.2 ml of aqueous 20% (v/v) ethanol. The combined extracts were treated with Amberlite CG 120 to remove excess [14 C]methylamine and analysed by chromatography and autoradiography as detailed in the Methods section.

Time (s)	Phosphates	Alanine	Malate	Aspartate	Glycine	Serine	Glutamate	Radioactivity (c.p.m.)*			Total
								Succinate +	Citrate +	Other compounds	
5	222	—	129	26	—	420	—	50	—	—	847
11	418	50	305	120	70	310	—	168	21	—	1462
16	847	120	295	184	138	244	63	166	92	—	2149
32	2584	300	389	244	326	536	520	206	652	567	6324

* Corrected for background = 60 c.p.m.

Table 2. Activities of key enzymes in *Pseudomonas* AM1 and *Pseudomonas* sp. MS grown on various substrates

The organisms were grown on the appropriate substrates until they were in the logarithmic growth phase. Organisms were harvested, extracts prepared and the enzyme activities measured as described in the Methods section.

Organism	Growth substrate	Enzyme activity (mu/mg of protein)				
		Hydroxypyruvate reductase	Phosphoenolpyruvate carboxylase	γ -Glutamylmethylamide synthetase	N-Methylglutamate synthetase	
<i>Pseudomonas</i> AM1	Methylamine	1930	120	0.047	0.090	
<i>Pseudomonas</i> AM1	Methanol	1615	210	0.042	< 0.06	
<i>Pseudomonas</i> AM1	Succinate	200	12	< 0.01	< 0.06	
<i>Pseudomonas</i> MS	Methylamine	< 1	15	1.70	7.32	
<i>Pseudomonas</i> MS	Trimethylsulphonium nitrate	< 1	32	0.075	0.133

Fig. 1. Variation with time of the percentage distribution of ^{14}C incorporated from $[^{14}\text{C}]$ methylamine into the constituents of the ethanol-soluble fraction of *Pseudomonas* AM1 growing on methylamine at 20°C . ▼, Phosphorylated compounds; ●, malate; △, aspartate; □, glycine; ▲, serine; ■, succinate+fumarate.

phosphorylated compounds and dicarboxylic acids. Upon further incubation (Fig. 1) the percentage of radioactivity found in serine decreased while that of malate and aspartate first increased and then decreased. These results are very similar to those obtained using $[^{14}\text{C}]$ methanol (Large *et al.* 1961). There was no evidence for any formation of either *N*-methylglutamate or γ -glutamylmethylamide.

Enzyme activities in *Pseudomonas* AM1 and *Pseudomonas* MS

Previous work has established the presence of high levels of hydroxypyruvate reductase in methanol- or formate-grown *Pseudomonas* AM1 (Large & Quayle, 1963). This enzyme is also present at high specific activity in extracts of methylamine-grown *Pseudomonas* AM1 (Table 2), whereas in methylamine-grown *Pseudomonas* MS no such activity could be detected. This contrasts with the distribution of *N*-methylglutamate synthetase and γ -glutamylmethylamide synthetase, both of which are virtually absent in *Pseudomonas* AM1 but present in methylamine-grown *Pseudomonas* MS. In trimethylsulphonium-grown *Pseudomonas* MS, however, none of the three enzymes was present in more than trace amounts.

A further difference between the two organisms is manifest in the activity of phosphoenolpyruvate carboxylase (Table 2). The specific activity of this enzyme in methylamine-

grown *Pseudomonas* MS is similar to that in succinate-grown *Pseudomonas* AMI, whereas growth of the latter organism on methanol or methylamine increases the specific activity 18- or 10-fold respectively.

DISCUSSION

The results obtained in this study confirm the predictions based on the growth characteristics of mutants that the pathway of carbon assimilation operating during growth of *Pseudomonas* AMI is the same on methylamine as on methanol or formate. There is no evidence either from the whole organism isotope experiment or the enzyme content of the organisms for the involvement of *N*-methylated amino acids as intermediary metabolites. On the basis of isotopic evidence, organism PAR also uses the serine pathway for growth on methylamine (Leadbetter & Gottlieb, 1967).

It is clear that *Pseudomonas* MS grows on methylamine by a pathway which differs in several respects from the serine pathway. The initial fixation of methylamine carbon results in methylation of glutamate to *N*-methylglutamate and γ -glutamylmethylamide. If these *N*-methylated amino acids function merely as intermediate C_1 carriers, the crucial steps of net biosynthesis being similar to those in *Pseudomonas* AMI, the fact that *Pseudomonas* MS lacks hydroxypyruvate reductase would point to a difference in the way in which the carbon skeleton of serine is converted to glycolytic intermediates such as phosphoglycerate.

Pseudomonas MS grown on trimethylsulphonium salts differed again. Hornig & Wagner (1968) showed that incubation of trimethylsulphonium-grown *Pseudomonas* MS with [^{14}C]trimethylsulphonium chloride resulted in early labelling of serine, glycine and alanine; no radioactive *N*-methylglutamate or γ -glutamylmethylamide was detected. The absence of the *N*-methylated amino acids is consistent with the negligible activities of *N*-methylglutamate synthetase and γ -glutamylmethylamide synthetase in extracts. As in the case of the methylamine-grown *Pseudomonas* MS no hydroxypyruvate reductase could be detected in the organism when grown on trimethylsulphonium salts. It is not clear from these data to what extent the carbon assimilatory pathways differ for methylamine and trimethylsulphonium salts in *Pseudomonas* MS. It would, however, be remarkable if the organism possessed two pathways of C_1 assimilation which were fundamentally, rather than superficially, different.

This study was supported in part by Grant no. GB-6084 from the National Science Foundation, U.S.A., to C.W. We thank Mr A. A. Hancock for his skilled technical assistance.

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